



Aungraheeta, R., Butler, M., Kelly, E., Nylander, S., Mumford, A., Mundell, S., & Conibear, A. E. (2016). Inverse agonism at the P2Y₁₂ receptor and ENT1 transporter blockade contribute to platelet inhibition by ticagrelor. *Blood*, 128(23), 2717-2728.
<https://doi.org/10.1182/blood-2016-03-707844>

Peer reviewed version

Link to published version (if available):
[10.1182/blood-2016-03-707844](https://doi.org/10.1182/blood-2016-03-707844)

[Link to publication record in Explore Bristol Research](#)
PDF-document

This is the author accepted manuscript (AAM). The final published version (version of record) is available online via ASH at <http://www.bloodjournal.org/content/early/2016/09/30/blood-2016-03-707844>. Please refer to any applicable terms of use of the publisher.

University of Bristol - Explore Bristol Research

General rights

This document is made available in accordance with publisher policies. Please cite only the published version using the reference above. Full terms of use are available:
<http://www.bristol.ac.uk/red/research-policy/pure/user-guides/ebr-terms/>

Inverse agonism at the P2Y₁₂ receptor and ENT1 transporter blockade contribute to platelet inhibition by ticagrelor

Running head: Ticagrelor and platelet signalling

Riyaad Aungraheeta¹, Alexandra Conibear¹, Mark Butler¹, Eamonn Kelly¹, Sven Nylander², Andrew Mumford³ and Stuart J.Mundell¹

¹ School of Physiology, Pharmacology and Neuroscience, Faculty of Biomedical Sciences, University of Bristol, Bristol, UK

² Cardiovascular and Metabolic Diseases, Innovative Medicines and Early Development Biotech Unit, AstraZeneca, Mölndal, Sweden

³ School of Clinical Sciences, Faculty of Health Sciences, University of Bristol, Bristol, UK

Correspondence: Stuart Mundell, School of Physiology, Pharmacology and Neuroscience, Faculty of Biomedical Sciences, University of Bristol, Bristol BS8 1TD, UK; email: s.j.mundell@bristol.ac.uk; Tel +44 (0) 117 3311440

Key Points

- Ticagrelor acts as an inverse agonist at the P2Y₁₂ receptor, inhibiting basal agonist-independent signalling.
- Ticagrelor inhibits the adenosine transporter ENT1 not only on erythrocytes, but on platelets too.

Abstract

Ticagrelor is a potent antagonist of the P2Y₁₂ receptor (P2Y₁₂R) and consequently an inhibitor of platelet activity effective in the treatment of atherothrombosis. Here, we sought to further characterise its molecular mechanism of action. Initial studies showed that ticagrelor promoted a greater inhibition of ADP-induced Ca²⁺ release in washed platelets versus other P2Y₁₂R antagonists. This additional effect of ticagrelor beyond P2Y₁₂R antagonism was in part as a consequence of ticagrelor inhibiting the equilibrative nucleoside transporter 1 (ENT1) on platelets, leading to accumulation of extracellular adenosine and activation of G_s-coupled adenosine A_{2A} receptors. This contributed to an increase in basal cAMP and VASP phosphorylation. In addition, ticagrelor increased platelet cAMP and VASP phosphorylation in the absence of ADP in an adenosine receptor-independent manner. We hypothesised that this increase originated from a direct effect on basal agonist-independent P2Y₁₂R signalling, and this was validated in 1321N1 cells stably transfected with human P2Y₁₂R. In these cells, ticagrelor blocked the constitutive agonist-independent activity of the P2Y₁₂R, limiting basal G_i-coupled signalling and thereby increasing cAMP levels. These data suggest that ticagrelor has the pharmacological profile of an inverse agonist. Based on our results showing insurmountable inhibition of ADP-induced Ca²⁺ release and forskolin-induced cAMP, the mode of antagonism of ticagrelor also appears non-competitive, at least functionally. In summary, our studies describe two novel modes of action of ticagrelor, inhibition of platelet ENT1 and inverse agonism at the P2Y₁₂R that contribute to its effective inhibition of platelet activation.

Introduction

Acute coronary syndrome (ACS) is amongst the leading causes of death in the world¹. Platelets play a pivotal role in the pathogenesis of ACS. Following atherosclerotic plaque rupture, platelets are exposed to potent agonists, collagen and thrombin, that trigger platelet activation and aggregation. Subsequent release of ADP from activated platelets and its activation of platelet P2Y₁₂Rs have a central role in amplifying the response to the initial stimulus. P2Y₁₂R signalling is therefore well established as a major positive feed-forward amplification mechanism to a wide variety of platelet agonists. Pharmacological blockade of the P2Y₁₂R represents an important and clinically well validated target for the treatment and prevention of thrombosis^{2,3}.

Unlike the thienopyridine P2Y₁₂R antagonists (ticlopidine, clopidogrel and prasugrel), ticagrelor binds to the P2Y₁₂R in a reversible manner^{4,5}. Also unlike the thienopyridines, which are all pro-drugs requiring metabolic activation to exert an antiplatelet effect, ticagrelor is direct acting. In addition, its main circulating metabolite, AR-C124910XX (present in plasma at 30-40% of parent⁶), has similar potency at the P2Y₁₂R as ticagrelor⁷. In comparison to clopidogrel, ticagrelor provides higher and more consistent platelet inhibition^{8,9}. Large clinical trials carried out across 43 countries in patients with ACS have demonstrated a lower rate of death due to adverse cardiovascular events overall, without an increase in serious bleeding in patients treated with ticagrelor compared to clopidogrel¹⁰. Ticagrelor is also superior to placebo when given on top of aspirin in patients with a prior myocardial infarction¹¹. Intriguingly, ticagrelor has recently been shown to inhibit the equilibrative nucleoside transporter 1 (ENT1)¹², an adenosine transporter, on red blood cells and thereby to increase extracellular adenosine levels *in vitro* and in the plasma of ticagrelor-treated patients¹³⁻¹⁶. Ticagrelor has also been shown to augment a number of physiological responses induced by adenosine, including increased coronary blood flow and adenosine-dependent inhibition of platelet aggregation^{15,17,18}. Some adverse effects associated with ticagrelor include dyspnoea and ventricular pauses, effects also seen with intracoronary administration of exogenous adenosine^{19,20}.

The aim of this study was to further elucidate the molecular mode of action of ticagrelor on platelets beyond its well-established antagonism of the P2Y₁₂R. Using isolated human platelets, we tested whether ticagrelor's inhibition of adenosine transport could contribute to changes in downstream signalling. We also verified whether these effects could be explained by the inhibition of platelet-expressed ENT1 by ticagrelor. We also tested the hypothesis that ticagrelor blocks constitutive agonist-independent P2Y₁₂R activity. The aim was to determine whether ticagrelor is an inverse agonist, unlike the active metabolite (R-138727) of the thienopyridine prasugrel, and thus help us gain a better comprehension of ticagrelor's efficacy.

Materials and Methods

Reagents

Membrane stripping solution and Fura-2 AM were from Thermo Fisher Scientific (Northumberland, UK). NECA, xanthine amine congener (XAC), AR-C 66096 tetrasodium salt, PSB 0739, 6-S-[(4-Nitrophenyl)methyl]-6-thioinosine (NBMPR) were procured from Tocris Bioscience (Bristol, UK). R-138727 was obtained from Eli Lilly Research Laboratories (Indianapolis, Indiana, USA). Ticagrelor was provided by AstraZeneca (Mölnådal, Sweden). Cell culture reagents included Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin-streptomycin and G418, which were from Invitrogen (Paisley, UK). Commercial antibodies used to detect Phospho-VASP (Ser157), VASP, Phospho-ERK, ERK were from New England Biolabs (Hertfordshire, UK), α -tubulin from Sigma Aldrich (Dorset, UK), ENT1 from Abcam (Cambridge, UK) and secondary mouse and rabbit horseradish peroxidase-conjugated antibodies were from GE Healthcare (Buckinghamshire, UK). All other reagents were purchased from Sigma-Aldrich (Dorset, UK).

Preparation of human platelets

Human blood was collected after informed consent from healthy, drug-free volunteers as described previously²¹. Platelets were rested for at least half an hour in the presence of 0.02U/ml apyrase and 10 μ M indomethacin prior to use in the experiments. All platelet preparations were conducted at room temperature and platelets were stored at 30°C.

Light transmission aggregometry

PRP samples stored at 30 °C were pre-heated to 37°C for 2 minutes and stirred at 1000 rpm in an aggregometer (Chronolog Model 700, Labmedics, Manchester, UK) before adding ADP (10 μ M). The change in optical density relative to PPP (100% aggregation reference) was recorded for 6 minutes and maximum aggregation extent was used for data analysis.

SDS-PAGE and immunoblotting

Protein samples were boiled in SDS sample buffer and subjected to Western analysis as previously described²². Where required, blots were stripped using Restore Western blot stripping solution (Thermo Scientific) and re-probed for protein of interest. Quantification was performed by densitometry using the ImageJ software (NIH, USA). Mean integrated density of bands of interest was used as a quantitative measurement of immunoblot intensity, divided by the mean integrated density of total protein (α -tubulin band), then normalised to the relevant control levels.

Measurement of cytosolic free Ca²⁺ in platelets

Measurement of intracellular Ca^{2+} release was adapted from a method used previously²². PRP was incubated with 4 μM Fura-2 AM (Life Technologies, Paisley, UK) in the presence of 0.02U/ml apyrase and 10 μM indomethacin at 30 °C for 1 hour. Platelets were pelleted by centrifugation at 550g for 10 minutes and resuspended in a modified Tyrode's-HEPES buffer (containing 0.02U/ml apyrase and 10 μM indomethacin) to a final concentration of 4 $\times 10^8$ /ml. Platelets were allowed to rest in the presence of vehicle (0.1% DMSO) or P2Y₁₂R antagonist for 30 minutes at 30°C. ADP (10 μM)-induced changes in Ca^{2+} levels were subsequently monitored at 37°C using a Tecan Infinite M200 PRO microplate reader (Labtech International Ltd, East Sussex, UK), with fluorescence excitation set at 340 and 380 nm, and emission at 510nm.

Measurement of platelet cAMP levels

Washed platelets were stimulated in the presence of the phosphodiesterase inhibitor IBMX (100 μM) and drug induced changes in platelet cAMP levels assessed as described before²³.

Cell culture

1321N1 cells stably expressing either haemagglutinin (HA)-tagged P2Y₁₂ or pcNEO vector were maintained in DMEM supplemented with 10% FCS, 100 units/ml penicillin-streptomycin and 400 μg /ml G418 (Geneticin) at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. The cloning and stable expression of P2Y₁₂ tagged at the N-terminus with HA in 1321N1 cells has previously been described²³.

Measurement of P2Y₁₂R activity in 1321N1 cells

P2Y₁₂R-stimulated inhibition of forskolin-stimulated adenylyl cyclase was measured in 1321N1 cells as previously described²³. Changes in cell VASP-P levels were assessed by immunoblotting as described above. Drug-induced changes in cell cAMP levels were determined using a cAMP Enzyme Immunoassay Kit according to the manufacturer's instructions.

Statistical analysis

Graphs were generated using Graphpad Prism (San Diego, CA, USA). Concentration-response curves were fitted to the data via a variable slope model of non-linear regression. Data points are displayed as mean \pm standard error. One-way analysis of variance (ANOVA), unless otherwise specified, was used to detect statistically significant differences and Bonferroni *post-hoc* analysis was applied.

Results

Ticagrelor-mediated inhibition of platelet Ca^{2+} release is partly adenosine receptor-dependent

Ticagrelor, AR-C 66096 and the active metabolite of prasugrel, R-138727, all inhibited ADP (10 μM)-induced platelet aggregation in human PRP in a concentration-dependent manner (Figure 1A). Ticagrelor (10 μM) significantly decreased the peak Ca^{2+} response to ADP (10 μM ; Figure 1B) with no evident change in the overall shape of the calcium trace versus that seen with agonist alone (data not shown). The P2Y₁₂R antagonists AR-C 66096 (10 μM) and R-138727 (10 μM) also inhibited Ca^{2+} release, although to a lesser extent than ticagrelor, as evident by the higher ADP-mediated maximum effect (E_{max}) values (Table 1).

Pre-incubation with the adenosine receptor antagonist xanthine amine congener (XAC; 10 μM) partly reversed the peak Ca^{2+} response in ticagrelor-treated platelets, to levels comparable to Ca^{2+} responses measured in AR-C 66096- and R-138727-treated platelets (Figure 1C). XAC (10 μM) treatment had no further effect on AR-C 66096- and R-138727-treated platelets (data not shown).

Ticagrelor induces an adenosine receptor-dependent increase in VASP-P in platelets

Incubation of washed platelets with ticagrelor induced a time- and concentration-dependent increase in vasodilator-stimulated phosphoprotein phosphorylation (VASP-P) in the absence of platelet agonist (Figure 2A-D). Ticagrelor (10 μM) induced a significant increase in VASP-P levels by 30 minutes with an upward trend at earlier time points (Figure 2A-B). Washed platelets incubated for a fixed time period (60 min) with a range of ticagrelor concentrations (0.1-10 μM) showed significant increases in VASP-P levels above basal, with concentrations greater than 3 μM (Figure 2C-D). Unlike ticagrelor (10 μM) none of the three alternative P2Y₁₂R antagonists, AR-C 66096, PSB 0739 and R-138727 (10 μM) were able to increase VASP-P beyond basal levels after incubation with platelets for 60 minutes (Figure 2E-F).

Similar to ticagrelor, the non-selective adenosine receptor agonist, NECA (1 μM ; 60 min) induced an increase in VASP-P (Figure 2G-H). Pre-incubating washed platelets with the unselective adenosine receptor antagonist XAC (1 μM ; 30 min) attenuated, but did not completely abolish subsequent ticagrelor (10 μM ; 60 min)- or NECA- (10 μM ; 60 min) induced increases in VASP-P (Figure 2G-H). The inhibitory effect of XAC was numerically but not significantly greater in combination with NECA as compared to ticagrelor. The adenosine receptor-dependent increase in VASP-P by ticagrelor was also observed in the presence of 1mM extracellular Ca^{2+} (data not shown). Pre-incubation with the A_{2A} adenosine receptor-selective antagonist SCH 442416 (1 μM ; 30 min) significantly attenuated ($p < 0.05$; $n = 6$) subsequent ticagrelor (10 μM ; 60 min)-induced increases in VASP-P whereas the A_{2B} adenosine receptor selective antagonist PSB 603 (1 μM ; 30 min) had no effect. VASP-P levels (fold over basal) were 9.19 ± 2.98 , 0.92 ± 0.37 and 8.54 ± 3.97 for ticagrelor alone, ticagrelor + SCH 442416 and ticagrelor + PSB 603 respectively).

Ticagrelor-mediated inhibition of platelet ENT1 likely explain adenosine-related effects

Incubation of washed platelets with the ENT1-specific inhibitor, NBMPR (1 μ M), induced a time-dependent increase ($p < 0.01$) in VASP-P (Figure 3A-B). This increase was comparable to that seen with ticagrelor (10 μ M). NBMPR in combination with ticagrelor did not significantly increase VASP-P compared to ticagrelor or NBMPR alone (Figure 3C-D). Pre-incubation of platelets with adenosine deaminase (ADA), which degrades adenosine into inosine, together with NBMPR or ticagrelor, prevented both NBMPR- ($p < 0.01$) and ticagrelor-mediated ($p < 0.01$) increases in VASP-P. Direct evidence of the presence of ENT1 in human platelets was obtained by immunoblotting (Figure 3E) using an ENT1-specific antibody.

Ticagrelor induces an adenosine receptor-independent increase in VASP-P and cAMP in platelets

XAC pre-incubation (10 μ M) reduced basal VASP-P (Figure 4A and Figure 4C), supporting the presence of endogenous adenosine and adenosine receptor signalling in our washed platelet preparations. In the presence of XAC, ticagrelor (10 μ M) produced a time- (Figure 4A-B) and concentration-dependent (Figure 4C-D) increase in VASP-P. The adenosine receptor agonist NECA (1 μ M; Figure 4E-F) and the P2Y₁₂R antagonists AR-C 66096, PSB 0739 and R-138727 (all at 10 μ M) however, did not affect VASP-P levels under the same conditions (Figure 4E-F). Ticagrelor was also still able to increase VASP-P in the presence of AR-C 66096, PSB 0739 or R-138727 (10 μ M; Figure 5A-B).

PGE₁ (1 μ M), a well-established stimulator of cAMP production through activation of platelet prostanoid receptors, increased cAMP levels which was attenuated by P2Y₁₂R activation with ADP (10 μ M; Figure 5C). As expected, the P2Y₁₂R antagonists ticagrelor, AR-C 66096 and R-138727 (10 μ M) attenuated the ADP-mediated reduction in cAMP levels (Figure 5C). However, only ticagrelor (10 μ M; 60 min) could modulate basal cAMP levels as evident by a 2-fold increase in cAMP ($p < 0.05$). Pre-incubation with XAC (10 μ M; 30 min) (Figure 5D) partially attenuated this ticagrelor-induced increase in cAMP. However, even in the presence of XAC, ticagrelor still significantly increased platelet cAMP levels, indicative of an adenosine-independent component in addition to the adenosine-dependent effect.

Ticagrelor also induces an adenosine receptor-independent increase in VASP-P in 1321N1 cells expressing the P2Y₁₂R

Given that ticagrelor increased basal platelet cAMP and VASP-P levels in the absence of adenosine receptor signalling, we hypothesised that ticagrelor was directly modulating agonist-independent P2Y₁₂R activity. In order to test this, we used P2 receptor-null 1321N1 human astrocytoma cells overexpressing the human P2Y₁₂R^{24,25}.

Pre-incubation with IBMX (100 μ M; 15 min), a phosphodiesterase inhibitor preventing enzymatic breakdown of cAMP, increased VASP-P as did forskolin (100nM; 5 min), a direct

activator of adenylyl cyclase. Forskolin-stimulated increases in VASP-P were, as expected, attenuated by P2Y₁₂R stimulation with ADP (10μM; 5 min). Ticagrelor (10μM; 5 min) increased VASP-P levels above basal whilst the other P2Y₁₂ antagonists AR-C 66096 and R-138727 (10μM; 5 mins) produced more modest effects (Figure 6A-B).

The effect of ticagrelor on the phosphorylation state of extracellular signal-regulated kinase (ERK) and Akt/protein kinase B, both shown to be activated downstream of P2Y₁₂R activation^{26,27} was explored. Ticagrelor produced no significant changes in ERK (Figure 6C-D) or Akt (data not shown) phosphorylation in P2Y₁₂R expressing cells.

Ticagrelor had no effect on VASP-P in pcNEO (vehicle vector)-transfected P2Y receptor-null cells, supporting a P2Y₁₂-dependent effect. Further, in P2Y₁₂R-expressing cells, pre-treatment with XAC (1μM; 15 mins) had no effect on ticagrelor-stimulated changes in VASP-P (Figure 6E-F), supporting an adenosine-independent effect.

Ticagrelor decreases the agonist-independent activity of the P2Y₁₂R

As expected and previously demonstrated, ADP produced a concentration-dependent decrease in forskolin-stimulated adenylyl cyclase activity (Figure 7A-B). Pre-treatment with either ticagrelor (Figure 7A) or AR-C 66096 (Figure 7B) both shifted the concentration-response curve of ADP in a parallel manner. However, the highest concentration of ticagrelor (30nM) reduced the maximal effect of the agonist ADP (Figure 7A). Schild-plot analysis of the shifts in ADP EC₅₀ produced by increasing concentrations of ticagrelor revealed a slope of 1.73±0.12 and a pA₂ of 8.79±0.31. Meanwhile at all concentrations of AR-C 66096, antagonism was surmountable (Figure 7B). Schild-plot analysis of the respective shifts of in ADP EC₅₀ concentrations gave a slope of close to unity (1.01±0.10) and a pA₂ of 8.2±0.22. Our Schild analysis therefore suggests that, ticagrelor and AR-C 66096 are acting as non-competitive and competitive antagonists respectively, with pA₂ values in broad agreement to those reported²⁸.

Upon closer examination of forskolin-stimulated adenylyl cyclase activity, a significant reduction was noted in P2Y₁₂R-expressing cells versus vector control cells (Figure 7D), indicating constitutive (agonist-independent) activity of the P2Y₁₂R. This attenuation of forskolin responsiveness due to P2Y₁₂R expression was reversed by ticagrelor (10μM; 30 min) but not with the other P2Y₁₂R antagonists (Figure 7E). The ability of ticagrelor to modulate this agonist-independent P2Y₁₂R activity was concentration-dependent (Figure 7F) and not affected by XAC pre-incubation (1μM; 15 min; data not shown). In summary, this set of results from 1321N1 cells indicated that ticagrelor was able to reduce agonist-independent P2Y₁₂R activity by acting as an inverse agonist.

Discussion

The P2Y₁₂R plays a central role in platelet activation and thrombosis, and has been by far the most successful target for antiplatelet therapy^{2,10,17,29-31}. Ticagrelor is the first non-thienopyridine P2Y₁₂ antagonist approved for the prevention of thrombotic events in ACS patients. Unlike the thienopyridines, ticagrelor is direct-acting and binds to P2Y₁₂ in a reversible manner to block platelet activity. Clinical studies provide evidence that ticagrelor treatment can offer superior efficacy compared to clopidogrel, with a lower mortality rate resulting from vascular causes, myocardial infarction or stroke.. The reasons for this are unclear but have recently been reviewed⁷. This study aimed to fully characterise how ticagrelor modulates human platelet function and demonstrates two novel antiplatelet mechanisms. Firstly, ticagrelor is able to directly block platelet ENT1 activity, leading to a rise in local extracellular adenosine levels, which in turn activates platelet A_{2A} adenosine receptors, increasing platelet cAMP levels and reducing platelet activity. Secondly, ticagrelor inhibits agonist-independent platelet P2Y₁₂R activity, which indicates that ticagrelor has the pharmacological profile of an inverse agonist. These effects are in addition to ticagrelor's primary antiplatelet mechanism of action, P2Y₁₂ antagonism.

A number of recent studies have shown that ticagrelor can increase extracellular adenosine levels^{15,16}. Experiments in whole blood demonstrated that ticagrelor blocked ENT1 adenosine transporters on red blood cells, resulting in increased plasma adenosine levels and inhibition of platelet aggregation¹⁵. However, a direct effect on platelet adenosine transport has not yet been demonstrated. In order to investigate potential effects on platelet signalling, we examined changes in platelet ADP-induced Ca²⁺ release, basal cAMP and basal VASP-P levels. We show that ticagrelor was more effective at reducing ADP-induced platelet Ca²⁺ release than other P2Y₁₂ antagonists with an evident decrease in agonist-stimulated peak Ca²⁺ concentrations but no overall change in the shape of the Ca²⁺ trace. The antagonism of the Ca²⁺ responses by ticagrelor, R-138727 and AR-C 66096 was insurmountable. For R-138727, this could be considered as consistent with it being an irreversibly-binding antagonist. Ticagrelor³² and the ATP analogue, AR-C 66096³³ however, reversibly bind to the P2Y₁₂R and yet displayed insurmountable antagonism.

In addition, ticagrelor treatment, unlike other P2Y₁₂ antagonists produced a time- and concentration-dependent increase in basal VASP-P in human washed platelets. Importantly, pre-treatment of platelets with the adenosine receptor antagonist XAC attenuated but did not abolish the ticagrelor-induced increase in VASP-P. This effect was independent of the presence of extracellular Ca²⁺. Although platelets express the A_{2A} adenosine receptor subtype and at lower levels the A_{2B} subtype^{34,35}, only the A_{2A} adenosine receptor-selective antagonist SCH 442416 attenuated the ticagrelor-induced increase in platelet VASP-P.

Our study is the first to demonstrate that ticagrelor is able to inhibit platelet-expressed ENT1. Previously, it has been shown that ticagrelor selectively inhibit cellular adenosine uptake via inhibition of ENT1 at clinically relevant concentrations¹² and that this inhibition translates into an increase in extracellular adenosine concentration^{15,16}. ENT1 on red blood cells are believed to play a key role because these cells are thought to be the main sink of adenosine in the circulation. However, this could not be applicable to our study since our

assays were carried out using washed platelets in the absence of erythrocytes. Proteomics databases (*e.g.* Platelet Web³⁶, ProteomicsDB³⁷) suggest that platelets do express ENT1 whilst our studies used Western blotting to confirm for the first time that platelets express ENT1. The identified protein was approximately 50 kDa in size, similar to that observed by other groups^{38,39}. Importantly, this band was not found in a mixed culture of rat glial cells which are reported to have little or no ENT1 expression⁴⁰. Further, the ENT1-selective inhibitor NBMPR, like ticagrelor, caused a time-dependent increase in VASP-P in washed platelets. Likewise, VASP-P peaked after a 60-minute incubation period with NBMPR or ticagrelor, indicative of a gradual build-up of adenosine after the adenosine transporter ENT1 was blocked. Hence following ticagrelor treatment, it would appear that inhibition of platelet as well as red blood cell ENT1 contribute to an increase in extracellular adenosine levels in blood.

Our results prompt us to speculate on the source of adenosine in our studies. Adenosine may be generated following ATP or ADP breakdown during preparation of washed platelets, which is undertaken in the presence of apyrase to preserve P2Y₁R and P2Y₁₂R responsiveness²³. It is well established that platelets express CD39/nucleoside triphosphate diphosphohydrolase 1 (NTPDase1), an ecto-nucleosidase enzyme that hydrolyses both ADP and ATP to AMP^{41,42}. Another platelet membrane-anchored enzyme, CD73 then converts AMP into adenosine^{43,44}. Critically, addition of adenosine deaminase which degrades adenosine into inosine, effectively blocked any adenosine-dependent effects of ticagrelor. Therefore as in previous studies, our data supports the fact that that ticagrelor does not directly bind to or stimulate platelet adenosine receptors but induces adenosine effects by increasing extracellular adenosine levels^{12,45}.

Our studies also show for the first time that ticagrelor can affect agonist-independent platelet cAMP and VASP-P, likely via P2Y₁₂R. This effect was adenosine receptor-independent as ticagrelor could still increase levels of platelet cAMP and VASP-P in the presence of adenosine receptor antagonism with XAC. The kinetics of the rise in VASP-P following XAC-induced adenosine receptor antagonism was notably faster compared to that in the absence of XAC. We hypothesise that the slow rise of VASP-P can be attributed to the gradual build-up of adenosine. In contrast, the rapid change in VASP-P apparent following adenosine receptor antagonism is suggestive of an effect consequent to direct receptor-ligand interaction. We suggest that ticagrelor is acting as an inverse agonist at the P2Y₁₂R, therefore having the opposite effect to ADP. This property again appears unique to ticagrelor versus the other P2Y₁₂R antagonists tested (Figure 4G-H).

Our hypothesis was validated in 1321N1 cells stably expressing the human P2Y₁₂R. Ticagrelor increased VASP-P only in 1321N1 cells expressing the P2Y₁₂R and not in P2Y null vector controls. In addition, we showed that XAC pre-incubation had no effect on ticagrelor-induced increase in VASP-P, indirectly supporting a selective effect on the P2Y₁₂R. Intriguingly, unlike in platelets, R-138727 but not AR-C 66096 also marginally increased VASP-P. Therefore, R-138727 may potentially also effect agonist-independent P2Y₁₂R activity. However, as we did not see any measurable effect on VASP-P in human platelets, or changes in forskolin responsiveness (see below), we did not explore this further.

It is also worth noting that ticagrelor did not affect ERK (Figure 6C-D) or Akt activation (data not shown) which are both phosphorylated following P2Y₁₂R activation⁴⁶. Closer examination of P2Y₁₂R signalling in 1321N1 cells showed that the receptor exhibited a high degree of constitutive activity visualised by an attenuation of forskolin responsiveness only in P2Y₁₂R-expressing cells⁴⁷. Pre-incubation with apyrase to remove residual ADP or pre-incubation with other P2Y₁₂R antagonists had no effect on forskolin responsiveness, providing further evidence that the receptor is able to initiate signalling in the absence of an agonist. Only ticagrelor was able to suppress agonist-independent activity of the P2Y₁₂R, as indicated by increased forskolin-induced cAMP levels in receptor-expressing cells to a level comparable with non-receptor-expressing cells. Our data provides compelling evidence that ticagrelor is an inverse agonist, stabilising the receptor in an inactive conformation upon binding and preventing agonist-independent coupling to G_i^{48,49}. We do recognise that these studies were undertaken in cell lines overexpressing the P2Y₁₂R (approximately 10 times greater than what we find in human platelets⁵⁰). Although receptor overexpression can affect the degree of agonist-independent activity, it is reassuring that we find comparable changes in the ability of ticagrelor to modulate basal P2Y₁₂R activity when the receptor is endogenously expressed on human platelets. Importantly, the “ionic lock” between the bottom of transmembrane domains III and VI seen in some G protein-coupled receptors (GPCRs), and which is thought to stabilise the inactive conformation of a GPCR in the absence of agonist ligand, is not present in the P2Y₁₂R⁵¹.

Another noteworthy finding from our studies in platelets was that pre-treatment with other P2Y₁₂R antagonists failed to attenuate adenosine receptor-independent ticagrelor-stimulated changes in basal VASP-P (Figure 5A-B). This suggests that AR-C 66096, PSB 0739 and R-138727, reported to bind to or close to the orthosteric/ADP binding site⁵¹⁻⁵³ did not prevent ticagrelor from binding to the P2Y₁₂R. A possible explanation is that ticagrelor binds to different residues to where the other antagonists bind. Closer examination of the 2MeSADP (agonist)-bound state crystal structure of the P2Y₁₂R revealed that ticagrelor cannot dock inside the 2MeSADP-binding cavity, whereas ADP and some P2Y₁₂R antagonists, including AR-C 66096 do have access⁵¹. Ticagrelor’s bulky side chains on N⁶ have been suggested to cause a steric clash, with docking likely requiring P2Y₁₂R helical rearrangements⁵. A study assessing P2Y₁₂R function in CHO cells indicated that ticagrelor acted in a competitive manner based on functional readouts although only low concentrations of ticagrelor (<30nM) were included in the analysis²⁸. In conflicting studies, ticagrelor has been shown to display characteristics of a non-competitive antagonist, decreasing the maximum response (E_{max}) as well as right-shifting the ADP concentration-response curve⁴. Ticagrelor did not displace [³H]ADP from the receptor (K_i > 10μM) but displaced 2MeS-ADP from membranes prepared from human washed platelets⁴. However, docking analysis would suggest that different binding sites for ADP and 2MeSADP are unlikely⁵¹. Relating to our platelet studies (Figure 1B) and Schild analysis in cell lines (Figure 7C), the data indicate that ticagrelor is acting in a non-competitive manner versus ADP. Due to the complex pharmacology of ticagrelor, it appears in our studies to behave as a non-competitive antagonist at a functional level although it may well bind to the orthosteric/ADP-binding site.

Only few inverse agonists are documented to act at the P2Y₁₂R⁵⁴. Ding *et al.* (2006) reported that AR-C78511 increased cAMP generation in cells expressing a chimeric P2Y₁₂R that displayed a high degree of constitutive activity, and is thus a potent inverse agonist⁵⁵. A follow-up study also revealed that AR-C78511 had superior antiplatelet activity compared to a neutral antagonist (cangrelor) in transgenic mice expressing the same chimeric receptor⁵⁶. Interestingly, AR-C78511, like ticagrelor, could not be docked to the 2MeSADP-bound conformation of the P2Y₁₂R, seemingly due to its own bulky N⁶ substituents⁵¹.

In summary, ticagrelor has a unique mode of action on human platelets, through blockade of agonist-dependent and independent P2Y₁₂R activity and through adenosine uptake inhibition. Notably these studies were carried out *ex vivo* after blood drawing with platelets isolated by centrifugation in the presence of anticoagulant with most assays carried out in the absence of erythrocytes and plasma. With these limitations of experimental design in mind the relative contribution of agonist-dependent and independent P2Y₁₂R activity in vivo to the clinical efficacy of ticagrelor remains to be determined.

Acknowledgements

The authors would like to thank the Bristol Platelet Group and other University of Bristol staff for kindly donating their blood and time. We are also grateful to Jonathan Hanley's lab for providing us with the rat glial cells.

Authorship contributions: RA, SM and AC designed and performed the research and wrote the manuscript. MB performed some of the research. EK, AM and SM designed and supervised the research. SN contributed to the manuscript.

Conflict of interest: SN is an employee of AstraZeneca.

Drug	E_{max} (AU)	<i>p</i>-value relative to control	<i>p</i>-value relative to ticagrelor	n
Vehicle control	0.92 [0.88 - 0.96]	NA	***	16
Ticagrelor	0.41 [0.38 – 0.45]	***	NA	22
AR-C 66096	0.61 [0.56 – 0.66]	***	***	12
R-138727	0.61 [0.57 – 0.66]	***	***	12

Table 1. Maximal responses (E_{max}) from ADP concentration vs. Ca²⁺ peak response curves (as calculated in GraphPad Prism) obtained in the presence of different P2Y₁₂R antagonists. Responses were recorded from washed platelets after incubation with vehicle (0.1% DMSO) or test compound (all at 10 μM; 30 minutes). 95% confidence for E_{max} values are shown in square brackets. *** indicates *p* < 0.001.

References

1. Pagidipati NJ, Gaziano TA. Estimating deaths from cardiovascular disease: a review of global methodologies of mortality measurement. *Circulation*. 2013;127(6):749-756.
2. Wallentin L, Becker RC, Budaj A, et al. Ticagrelor versus clopidogrel in patients with acute coronary syndromes. *N Engl J Med*. 2009;361(11):1045-1057.
3. Wiviott SD, Braunwald E, McCabe CH, et al. Prasugrel versus clopidogrel in patients with acute coronary syndromes. *N Engl J Med*. 2007;357(20):2001-2015.
4. JJ VANG, Nilsson L, Berntsson P, et al. Ticagrelor binds to human P2Y₁₂ independently from ADP but antagonizes ADP-induced receptor signaling and platelet aggregation. *J Thromb Haemost*. 2009;7(9):1556-1565.
5. Paoletta S, Sabbadin D, von Kugelgen I, et al. Modeling ligand recognition at the P2Y₁₂ receptor in light of X-ray structural information. *J Comput Aided Mol Des*. 2015;29(8):737-756.
6. Storey RF, Husted S, Harrington RA, et al. Inhibition of platelet aggregation by AZD6140, a reversible oral P2Y₁₂ receptor antagonist, compared with clopidogrel in patients with acute coronary syndromes. *J Am Coll Cardiol*. 2007;50(19):1852-1856.
7. Nylander S, Schulz R. Effects of P2Y receptor antagonists beyond platelet inhibition - comparison of ticagrelor with thienopyridines. *Br J Pharmacol*. 2016.
8. Aleil B, Ravanat C, Cazenave JP, Rochoux G, Heitz A, Gachet C. Flow cytometric analysis of intraplatelet VASP phosphorylation for the detection of clopidogrel resistance in patients with ischemic cardiovascular diseases. *J Thromb Haemost*. 2005;3(1):85-92.
9. Gurbel PA, Bliden KP, Butler K, et al. Response to ticagrelor in clopidogrel nonresponders and responders and effect of switching therapies: the RESPOND study. *Circulation*. 2010;121(10):1188-1199.
10. Lindholm D, Varenhorst C, Cannon CP, et al. Ticagrelor vs. clopidogrel in patients with non-ST-elevation acute coronary syndrome with or without revascularization: results from the PLATO trial. *Eur Heart J*. 2014;35(31):2083-2093.
11. Bonaca MP, Bhatt DL, Cohen M, et al. Long-term use of ticagrelor in patients with prior myocardial infarction. *N Engl J Med*. 2015;372(19):1791-1800.
12. Armstrong D, Summers C, Ewart L, Nylander S, Sidaway JE, van Giezen JJ. Characterization of the adenosine pharmacology of ticagrelor reveals therapeutically relevant inhibition of equilibrative nucleoside transporter 1. *J Cardiovasc Pharmacol Ther*. 2014;19(2):209-219.
13. Armstrong PC, Leadbeater PD, Chan MV, et al. In the presence of strong P2Y₁₂ receptor blockade, aspirin provides little additional inhibition of platelet aggregation. *J Thromb Haemost*. 2011;9(3):552-561.
14. van Giezen JJ, Sidaway J, Glaves P, Kirk I, Bjorkman JA. Ticagrelor inhibits adenosine uptake in vitro and enhances adenosine-mediated hyperemia responses in a canine model. *J Cardiovasc Pharmacol Ther*. 2012;17(2):164-172.
15. Nylander S, Femia EA, Scavone M, et al. Ticagrelor inhibits human platelet aggregation via adenosine in addition to P2Y₁₂ antagonism. *J Thromb Haemost*. 2013;11(10):1867-1876.
16. Bonello L, Laine M, Kipson N, et al. Ticagrelor increases adenosine plasma concentration in patients with an acute coronary syndrome. *J Am Coll Cardiol*. 2014;63(9):872-877.
17. Alexopoulos D, Moulias A, Koutsogiannis N, et al. Differential effect of ticagrelor versus prasugrel on coronary blood flow velocity in patients with non-ST-elevation acute coronary syndrome undergoing percutaneous coronary intervention: an exploratory study. *Circ Cardiovasc Interv*. 2013;6(3):277-283.
18. Cattaneo M, Schulz R, Nylander S. Adenosine-mediated effects of ticagrelor: evidence and potential clinical relevance. *J Am Coll Cardiol*. 2014;63(23):2503-2509.
19. Storey RF, Bliden KP, Patil SB, et al. Incidence of dyspnea and assessment of cardiac and pulmonary function in patients with stable coronary artery disease receiving ticagrelor, clopidogrel, or placebo in the ONSET/OFFSET study. *J Am Coll Cardiol*. 2010;56(3):185-193.

20. Storey RF, Becker RC, Harrington RA, et al. Characterization of dyspnoea in PLATO study patients treated with ticagrelor or clopidogrel and its association with clinical outcomes. *Eur Heart J*. 2011;32(23):2945-2953.
21. Barton JF, Hardy AR, Poole AW, Mundell SJ. Reciprocal regulation of platelet responses to P2Y and thromboxane receptor activation. *J Thromb Haemost*. 2008;6(3):534-543.
22. Mundell SJ, Barton JF, Mayo-Martin MB, Hardy AR, Poole AW. Rapid resensitization of purinergic receptor function in human platelets. *J Thromb Haemost*. 2008;6(8):1393-1404.
23. Hardy AR, Conley PB, Luo J, Benovic JL, Poole AW, Mundell SJ. P2Y1 and P2Y12 receptors for ADP desensitize by distinct kinase-dependent mechanisms. *Blood*. 2005;105(9):3552-3560. Epub 2005 Jan 3521.
24. Nisar SP, Cunningham M, Saxena K, Pope RJ, Kelly E, Mundell SJ. Arrestin scaffolds NHERF1 to the P2Y12 receptor to regulate receptor internalization. *J Biol Chem*. 2012;287(29):24505-24515.
25. Patel YM, Lordkipanidze M, Lowe GC, et al. A novel mutation in the P2Y12 receptor and a function-reducing polymorphism in protease-activated receptor 1 in a patient with chronic bleeding. *J Thromb Haemost*. 2014;12(5):716-725.
26. Kim S, Jin J, Kunapuli SP. Akt activation in platelets depends on Gi signaling pathways. *J Biol Chem*. 2004;279(6):4186-4195.
27. Soulet C, Sauzeau V, Plantavid M, et al. Gi-dependent and -independent mechanisms downstream of the P2Y12 ADP-receptor. *J Thromb Haemost*. 2004;2(1):135-146.
28. Hoffmann K, Lutz DA, Strassburger J, Baqi Y, Muller CE, von Kugelgen I. Competitive mode and site of interaction of ticagrelor at the human platelet P2Y12 -receptor. *J Thromb Haemost*. 2014;12(11):1898-1905.
29. Born GV. Adenosine diphosphate as a mediator of platelet aggregation in vivo: an editorial view. *Circulation*. 1985;72(4):741-746.
30. Alexopoulos D, Xanthopoulou I, Mavronasiou E, et al. Randomized assessment of ticagrelor versus prasugrel antiplatelet effects in patients with diabetes. *Diabetes Care*. 2013;36(8):2211-2216.
31. Alexopoulos D, Galati A, Xanthopoulou I, et al. Ticagrelor versus prasugrel in acute coronary syndrome patients with high on-clopidogrel platelet reactivity following percutaneous coronary intervention: a pharmacodynamic study. *J Am Coll Cardiol*. 2012;60(3):193-199.
32. Husted S, van Giezen JJ. Ticagrelor: the first reversibly binding oral P2Y12 receptor antagonist. *Cardiovasc Ther*. 2009;27(4):259-274.
33. Humphries RG, Tomlinson W, Ingall AH, Cage PA, Leff P. FPL 66096: a novel, highly potent and selective antagonist at human platelet P2T-purinoceptors. *Br J Pharmacol*. 1994;113(3):1057-1063.
34. Salama SE, Haslam RJ. Subcellular distribution of cyclic AMP-dependent protein kinase activity and of cyclic AMP-binding proteins in human platelets. Modification by Ca²⁺-dependent proteolysis. *FEBS Lett*. Vol. 130. Netherlands; 1981:230-234.
35. Dixon AK, Gubitzi AK, Sirinathsinghji DJ, Richardson PJ, Freeman TC. Tissue distribution of adenosine receptor mRNAs in the rat. *Br J Pharmacol*. 1996;118(6):1461-1468.
36. Boyanova D, Nilla S, Birschmann I, Dandekar T, Dittrich M. PlateletWeb: a systems biologic analysis of signaling networks in human platelets. *Blood*. 2012;119(3):e22-34.
37. Wilhelm M, Schlegl J, Hahne H, et al. Mass-spectrometry-based draft of the human proteome. *Nature*. 2014;509(7502):582-587.
38. Alanko L, Porkka-Heiskanen T, Soinila S. Localization of equilibrative nucleoside transporters in the rat brain. *J Chem Neuroanat*. 2006;31(3):162-168.
39. Musa H, Dobrzynski H, Berry Z, et al. Immunocytochemical demonstration of the equilibrative nucleoside transporter rENT1 in rat sinoatrial node. *J Histochem Cytochem*. 2002;50(3):305-309.
40. Guillen-Gomez E, Calbet M, Casado J, et al. Distribution of CNT2 and ENT1 transcripts in rat brain: selective decrease of CNT2 mRNA in the cerebral cortex of sleep-deprived rats. *J Neurochem*. 2004;90(4):883-893.

41. Koziak K, Seigny J, Robson SC, Siegel JB, Kaczmarek E. Analysis of CD39/ATP diphosphohydrolase (ATPDase) expression in endothelial cells, platelets and leukocytes. *Thromb Haemost.* 1999;82(5):1538-1544.
42. Marcus AJ, Safier LB, Hajjar KA, et al. Inhibition of platelet function by an aspirin-insensitive endothelial cell ADPase. Thromboregulation by endothelial cells. *J Clin Invest.* 1991;88(5):1690-1696.
43. Zimmermann H. 5'-Nucleotidase: molecular structure and functional aspects. *Biochem J.* 1992;285 (Pt 2):345-365.
44. Dwyer KM, Robson SC, Nandurkar HH, et al. Thromboregulatory manifestations in human CD39 transgenic mice and the implications for thrombotic disease and transplantation. *J Clin Invest.* 2004;113(10):1440-1446.
45. Teng R, Oliver S, Hayes MA, Butler K. Absorption, distribution, metabolism, and excretion of ticagrelor in healthy subjects. *Drug Metab Dispos.* 2010;38(9):1514-1521.
46. Kahner BN, Shankar H, Murugappan S, Prasad GL, Kunapuli SP. Nucleotide receptor signaling in platelets. *J Thromb Haemost.* 2006;4(11):2317-2326.
47. Schulz A, Schoneberg T. The structural evolution of a P2Y-like G-protein-coupled receptor. *J Biol Chem.* 2003;278(37):35531-35541.
48. Kenakin T. Efficacy as a vector: the relative prevalence and paucity of inverse agonism. *Mol Pharmacol.* 2004;65(1):2-11.
49. Kenakin T. Efficacy at G-protein-coupled receptors. *Nat Rev Drug Discov.* 2002;1(2):103-110.
50. Mundell SJ, Jones ML, Hardy AR, et al. Distinct roles for protein kinase C isoforms in regulating platelet purinergic receptor function. *Mol Pharmacol.* 2006;70(3):1132-1142.
51. Zhang J, Zhang K, Gao ZG, et al. Agonist-bound structure of the human P2Y12 receptor. *Nature.* 2014;509(7498):119-122.
52. Hoffmann K, Baqi Y, Morena MS, Glanzel M, Muller CE, von Kugelgen I. Interaction of new, very potent non-nucleotide antagonists with Arg256 of the human platelet P2Y12 receptor. *J Pharmacol Exp Ther.* 2009;331(2):648-655.
53. Algaier I, Jakubowski JA, Asai F, von Kugelgen I. Interaction of the active metabolite of prasugrel, R-138727, with cysteine 97 and cysteine 175 of the human P2Y12 receptor. *J Thromb Haemost.* 2008;6(11):1908-1914.
54. Schmidt P, Ritscher L, Dong EN, et al. Identification of Determinants Required for Agonistic and Inverse Agonistic Ligand Properties at the ADP Receptor P2Y12. *Mol Pharmacol.* 2013;83(1):256-266.
55. Ding Z, Kim S, Kunapuli SP. Identification of a potent inverse agonist at a constitutively active mutant of human P2Y12 receptor. *Mol Pharmacol.* 2006;69(1):338-345.
56. Zhang Y, Ye J, Hu L, et al. Increased platelet activation and thrombosis in transgenic mice expressing constitutively active P2Y12. *J Thromb Haemost.* 2012;10(10):2149-2157.

Figure 1. ADP-induced platelet aggregation and intracellular Ca²⁺ release

(A) ADP (10 μ M)-induced platelet aggregation in human PRP after 30-minute incubation at 30 °C with ticagrelor (●; IC₅₀ = 0.27 μ M [0.14 - 0.51]; n=3), AR-C 66096 (▼; IC₅₀ = 0.16 μ M [0.08 - 0.33] n=4) or R-138727 (▲; IC₅₀ = 3.82 μ M [3.03 - 4.82]; n=6). 95% confidence intervals for IC₅₀s are shown in square brackets. (B) ADP-induced Ca²⁺ peak responses in Fura-2 AM-loaded platelets in the presence of vehicle (○; n=16) or 10 μ M ticagrelor (●; n=22), AR-C 66096 (▼; n=12) or R-138727 (▲; n=12). Baseline readings were recorded over a period of 5 cycles (each cycle ~ 7 s) before ADP addition, after which recording was continued for a further 15 cycles. The peak Ca²⁺ response was determined by calculating the change in fluorescence ratio (340 nm/380 nm). (C) ADP (100 μ M)-induced platelet peak Ca²⁺ responses in the presence of vehicle (0.1% DMSO), ticagrelor (10 μ M), ticagrelor (10 μ M) + XAC (10 μ M), AR-C 66096 (10 μ M) and R-138727 (10 μ M). Data displayed as mean and SEM. * indicates $p < 0.05$.

Figure 2. Time- and concentration-dependence of increases in platelet VASP-P

Representative immunoblots and bar/x-y charts showing VASP-P, VASP and α -tubulin (internal control) levels in washed platelets after pre-incubation at 37 °C with: (A-B) 10 μ M ticagrelor for 0, 0.5, 5, 10, 15, 30 and 60 minutes; n=5. (C-D) vehicle (0.1% DMSO), 0.1, 0.3, 1, 3 and 10 μ M ticagrelor for 60 minutes; n=5. (E-F) untreated, vehicle (0.1% DMSO), ticagrelor (10 μ M), AR-C 66096 (10 μ M), PSB 0739 (10 μ M) and R-138727 (10 μ M) for 60 minutes; n=4. (G-H) untreated, vehicle (0.1% DMSO), ticagrelor (10 μ M) and NECA (μ M) for 60 minutes following pre-incubation at 37 °C for 30 minutes with: untreated, vehicle (0.1% DMSO) or XAC (1 μ M), n=6. Data displayed as mean and SEM. ** indicates $p < 0.01$; ***, $p < 0.001$; and ns, $p > 0.05$. Statistical analysis used was two-way ANOVA (Figure 2H). Kilodaltons of molecular size markers are reported on the left.

Figure 3. Role for platelet-expressed ENT1 in the ticagrelor-induced increase in platelet VASP-P

Representative immunoblots and bar charts showing VASP-P, VASP and α -tubulin (internal control) levels in washed platelets after incubation at 37 °C with: (A-B) no drug, vehicle (0.1% DMSO for 60 min), NBMPR (1 μ M for 0.5, 5, 10, 15, 30 and 60 min) or ticagrelor (10 μ M for 0.5, 5, 10, 15, 30 and 60 min); n=5. (C-D) vehicle (0.1% DMSO), ticagrelor (10 μ M), NBMPR (1 μ M), ticagrelor (10 μ M) + NBMPR (1 μ M), adenosine deaminase (10 μ g/ml), adenosine deaminase (10 μ g/ml) + ticagrelor (10 μ M) and adenosine deaminase + NBMPR (1 μ M); n=3. (E) Immunoblots showing expression of ENT1 in platelets and 1321N1 cells, with rat glial cells as negative control. Figure is representative of three replicates. Data displayed as mean and SEM. * indicates $p < 0.05$; **, $p < 0.01$; and ns, $p > 0.05$. Statistical test used was two-way ANOVA (Figure 3B). Kilodaltons of molecular size markers are reported on the left or in the middle for Figure G.

Figure 4. Identification of an adenosine receptor-independent component of the ticagrelor-induced increase in platelet VASP-P and cAMP

Representative immunoblots and bar/x-y charts showing VASP-P, VASP and α -tubulin (loading control) levels in washed platelets after incubation at 37 °C with: (A-B) vehicle (0.1% DMSO for 60 minutes) or 10 μ M ticagrelor for 0.5, 5, 10, 15, 30 and 60 minutes following pre-incubation of all samples (except vehicle control) with XAC (10 μ M, at 37 °C) for 90 minutes; n=3. (C-D) vehicle (0.1% DMSO), 0.1, 0.3, 1, 3 and 10 μ M ticagrelor for 60 minutes following pre-incubation of all samples (except vehicle control) with XAC (10 μ M at 37 °C) for 90 minutes; n=5. (E-F) no drug, vehicle (0.1% DMSO), ticagrelor (10 μ M) and NECA (1 μ M) for 60 minutes following pre-incubation of all samples (except vehicle control) with XAC (10 μ M at 37 °C) for 90 minutes; n=5. (G-H) vehicle (0.1% DMSO), ticagrelor (10 μ M), AR-C 66096 (10 μ M), PSB 0739 (10 μ M) and R-138727 (10 μ M) for 60 minutes * indicates $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ and ns, $p > 0.05$. Kilodaltons of molecular size markers are shown on left of blots.

Figure 5. Ticagrelor-mediated, adenosine receptor-independent increases in platelet VASP-P in the presence of other P2Y₁₂ antagonists

(A-B) Representative immunoblots and bar charts showing VASP-P, VASP and α -tubulin (internal control) levels in washed platelets after incubation (60 minutes) at 37 °C with vehicle (0.1% DMSO), ticagrelor (10 μ M) or NECA (1 μ M). Pre-incubation conditions were either vehicle (0.1% DMSO) + XAC (10 μ M), AR-C 66096 (10 μ M) + XAC (10 μ M), PSB 0739 (10 μ M) + XAC (10 μ M) or R-138727 (10 μ M) + XAC (10 μ M), all at 37 °C for 30 minutes; n=7. (C-D) cAMP accumulation in washed platelets. (C) Platelets were pre-incubated with either ticagrelor, AR-C 66096 and R-138727 (all at 10 μ M; 60 mins) or vehicle control. Platelets were stimulated with PGE₁ (1 μ M; 10 mins) in the absence or presence of ADP (10 μ M) with. (D) Effect of antagonists on basal cAMP levels. Incubation (60 minutes) at 37 °C with vehicle (0.1% DMSO), ticagrelor (10 μ M), PSB 0739 (10 μ M) or R-138727 (10 μ M) for 60 minutes and pre-incubation with XAC (10 μ M; 60 mins). All bar charts display mean and SEM. Statistical test used was two-way ANOVA (excluding NECA data) (Figure 5B). * indicates $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. Kilodaltons of molecular size markers are shown on blots.

Figure 6. Ticagrelor-mediated adenosine receptor-independent increases in VASP-P in 1321N1 cells expressing P2Y₁₂R.

Representative immunoblots and bar/x-y charts showing: (A-B) VASP-P, VASP and α -tubulin (internal control) levels in P2Y₁₂-transfected 1321N1 cells incubated with phosphodiesterase inhibitor IBMX (100 μ M), adenylyl cyclase activator forskolin (100nM), forskolin (100nM) + ADP (10 μ M), ADP (10 μ M), ticagrelor (10 μ M), AR-C 66096 (10 μ M) or R-138727 (10 μ M) for 5 minutes at 37 °C; n=5. (C-D) ERK-P, ERK and α -tubulin (internal control) levels in P2Y₁₂-transfected 1321N1 cells incubated with ticagrelor for 0, 5, 10, 15, 30 and 60 minutes, at 37 °C; n=5 (E-F) VASP-P and α -tubulin (internal control) levels in P2Y₁₂-transfected (E left) and vehicle vector pcNEO-transfected (E right) 1321N1 cells, both pre-incubated with either XAC (1 μ M) or vehicle (0.1% DMSO) for 15 minutes at 37 °C before incubation with ticagrelor (10 μ M) for 0, 5, 15, 30 and 60 minutes at 37 °C. All bar/x-y charts display mean and SEM. Statistical test used was two-way ANOVA (Figure 6D). * indicates $p < 0.05$; **, $p < 0.01$. Kilodaltons of molecular size markers are reported on the left.

Figure 7. Ticagrelor-mediated attenuation of agonist-dependent and –independent P2Y₁₂R activity in 1321N1 cells.

Inhibition of forskolin (1 μ M; 10 min)-induced cAMP production by ADP (0.1 nM – 100 μ M; 10 min) in P2Y₁₂R-transfected 1321N1 cells after pre-incubation with (A) ticagrelor (3-30 nM; 15 minutes) or (B) AR-C 66096 (AR-C; 3-30 nM; 15 min); n=5. (C) Schild-plot analysis of data from (A) and (B). (D) Forskolin (0.1 nM – 1 μ M; 10 min)-induced increases in cAMP levels in pcNEO-transfected and P2Y₁₂R-transfected 1321N1 cells. (E) Forskolin (1 μ M; 10 min)-induced cAMP production in pcNEO-transfected and P2Y₁₂-transfected cells incubated with vehicle (0.1% DMSO), ticagrelor (10 μ M), AR-C 66096 (10 μ M) or R-138727 (10 μ M) for 30 minutes. (F) Forskolin (1 μ M; 10 min)-induced cAMP after pre-incubation with vehicle (0.1% DMSO) or ticagrelor (0.01 μ M – 10 μ M; 30 min) in P2Y₁₂R-transfected cells; n=5. All graphs and bar charts display mean \pm SEM. All cells were incubated with 0.2 U/ml apyrase (37 °C; 60 min) prior to drug additions. * indicates $p < 0.05$ versus pcNEO controls.

Figure 1

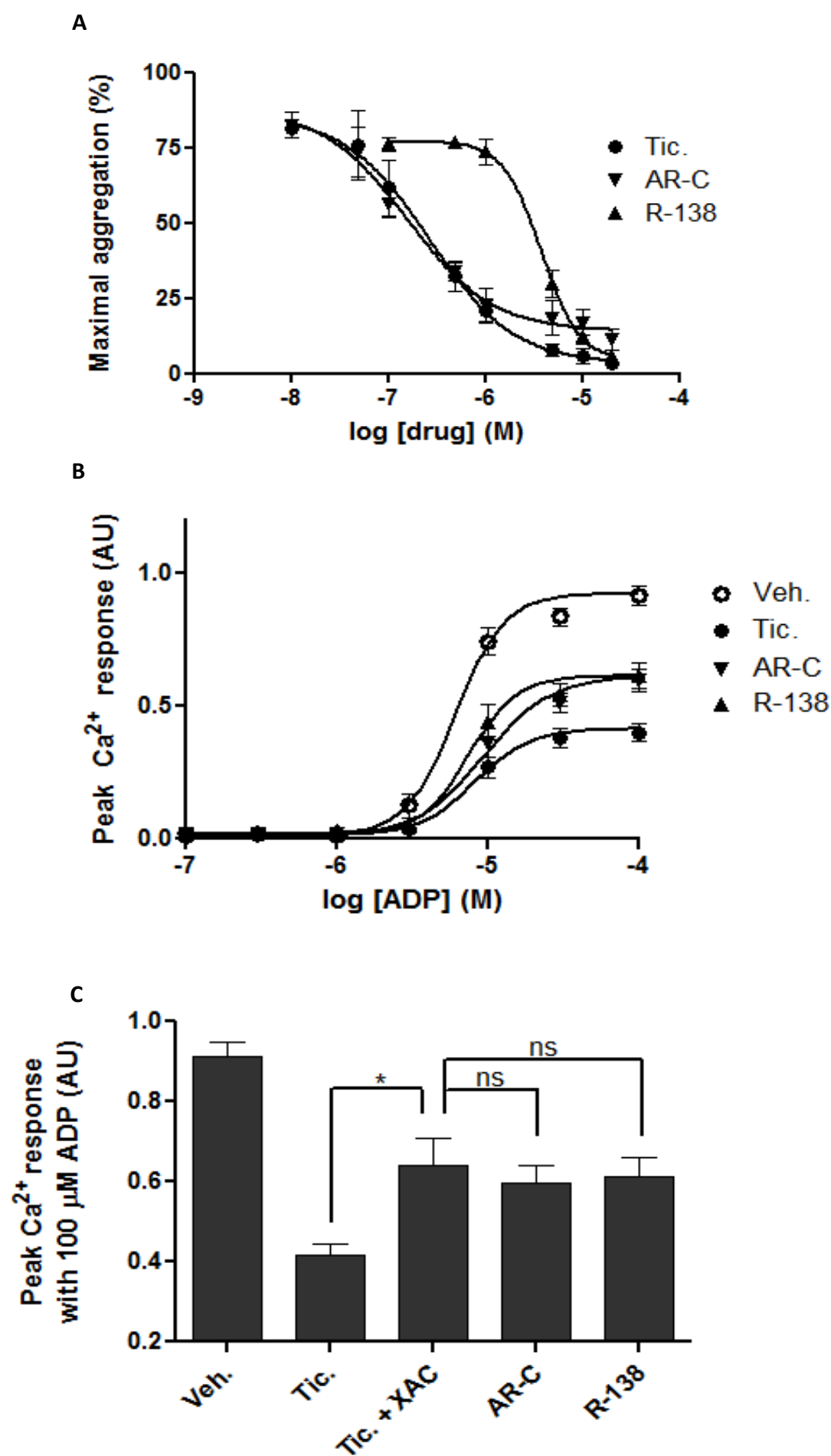


Figure 2

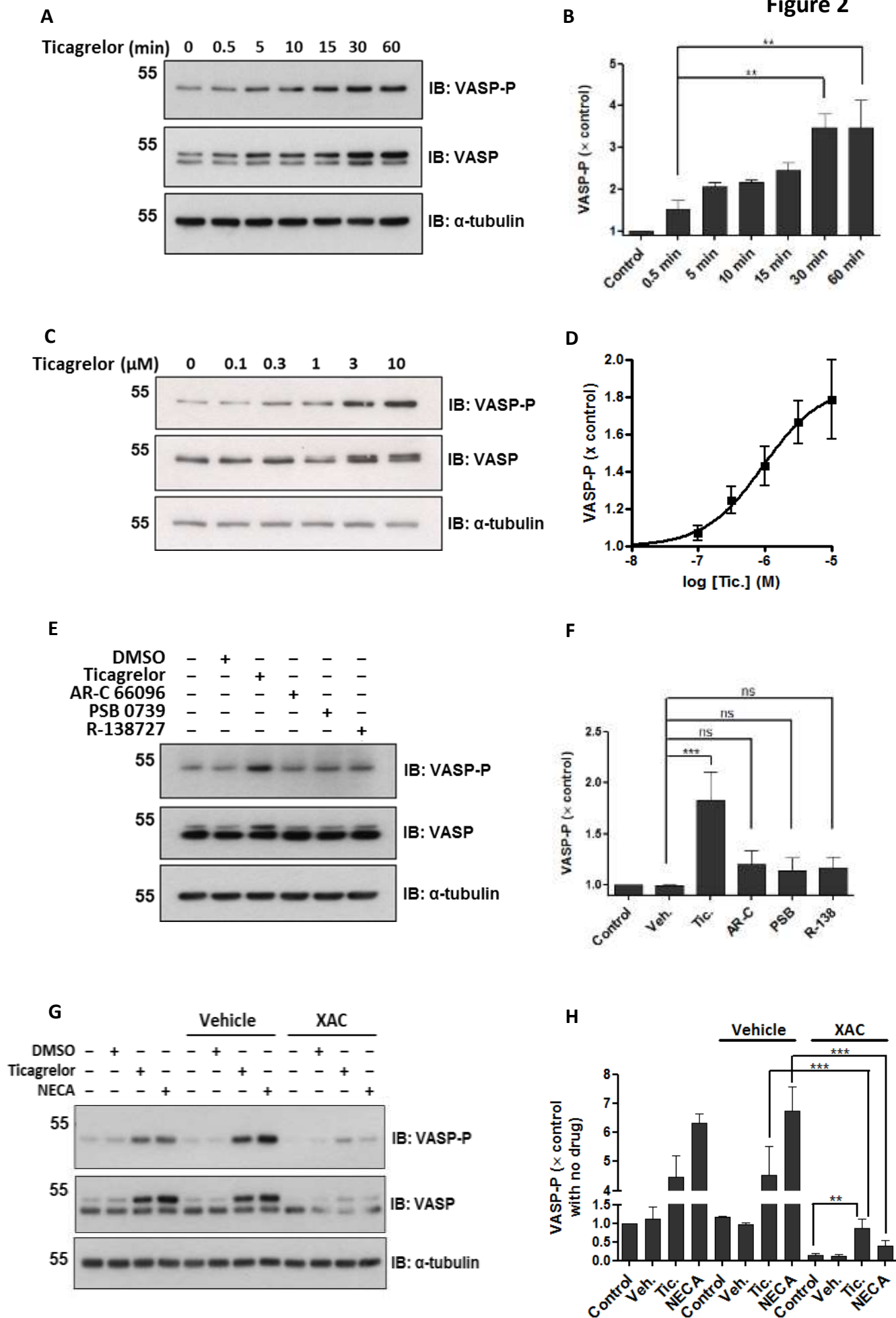


Figure 3

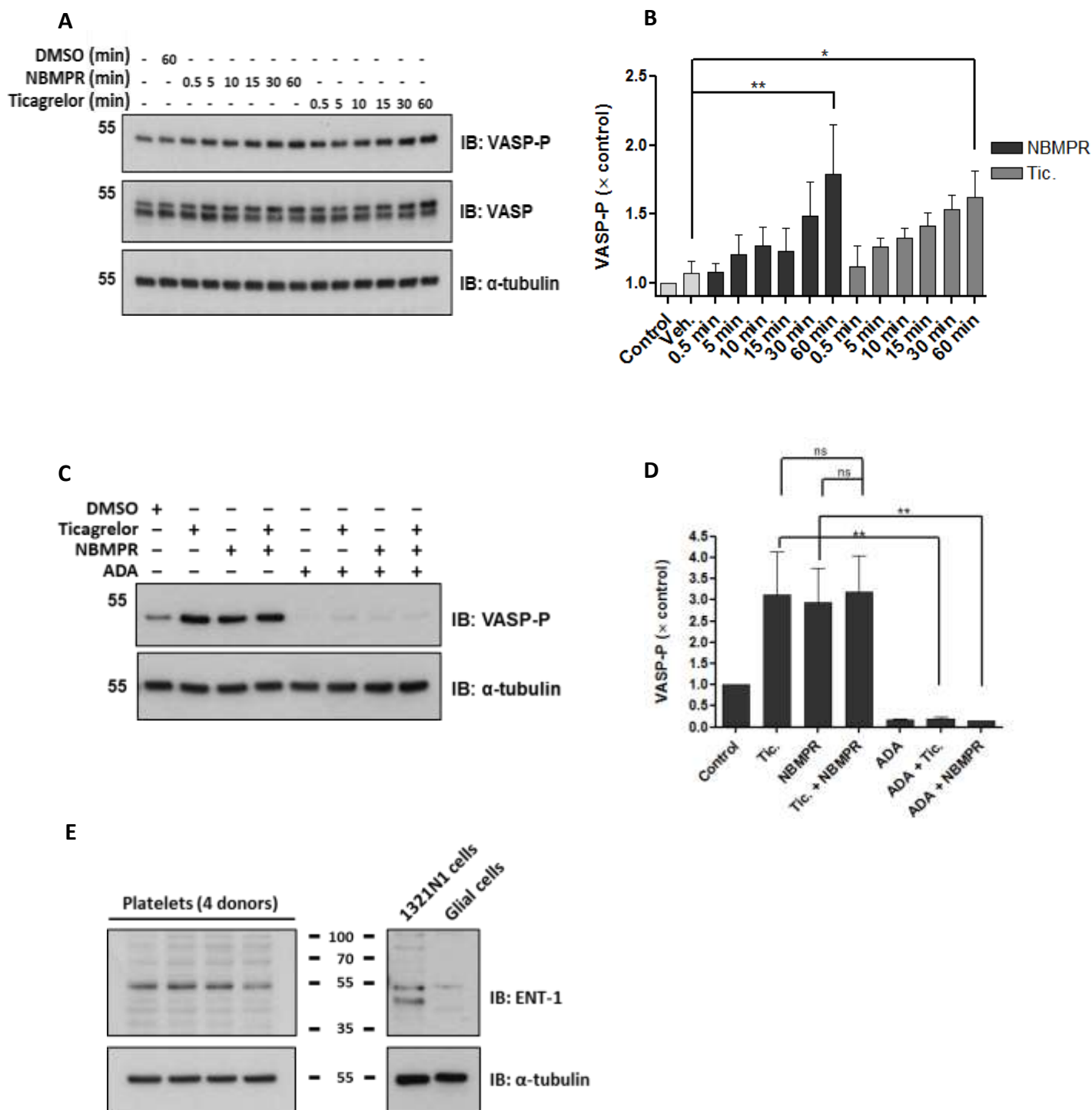


Figure 4

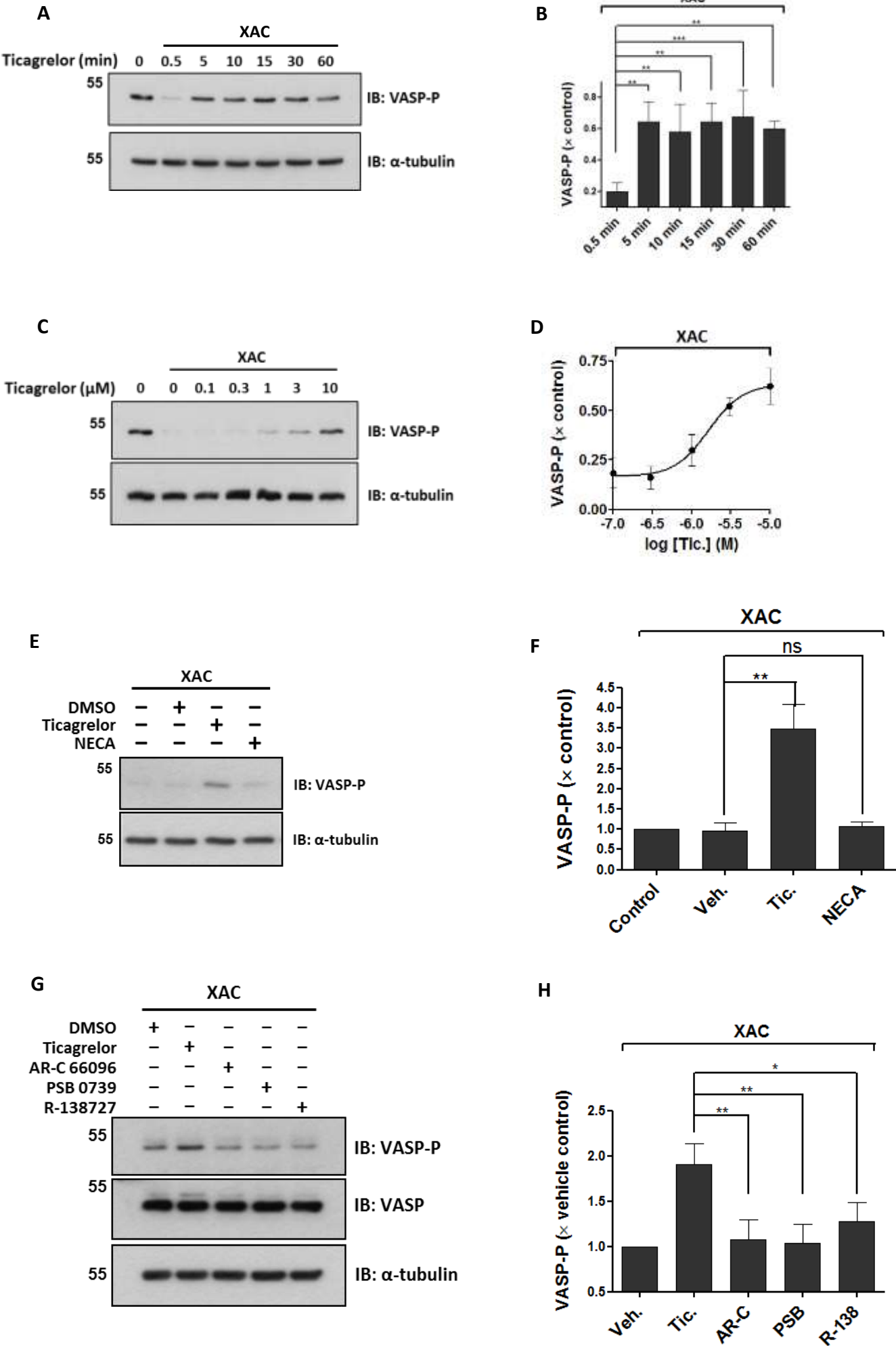


Figure 5

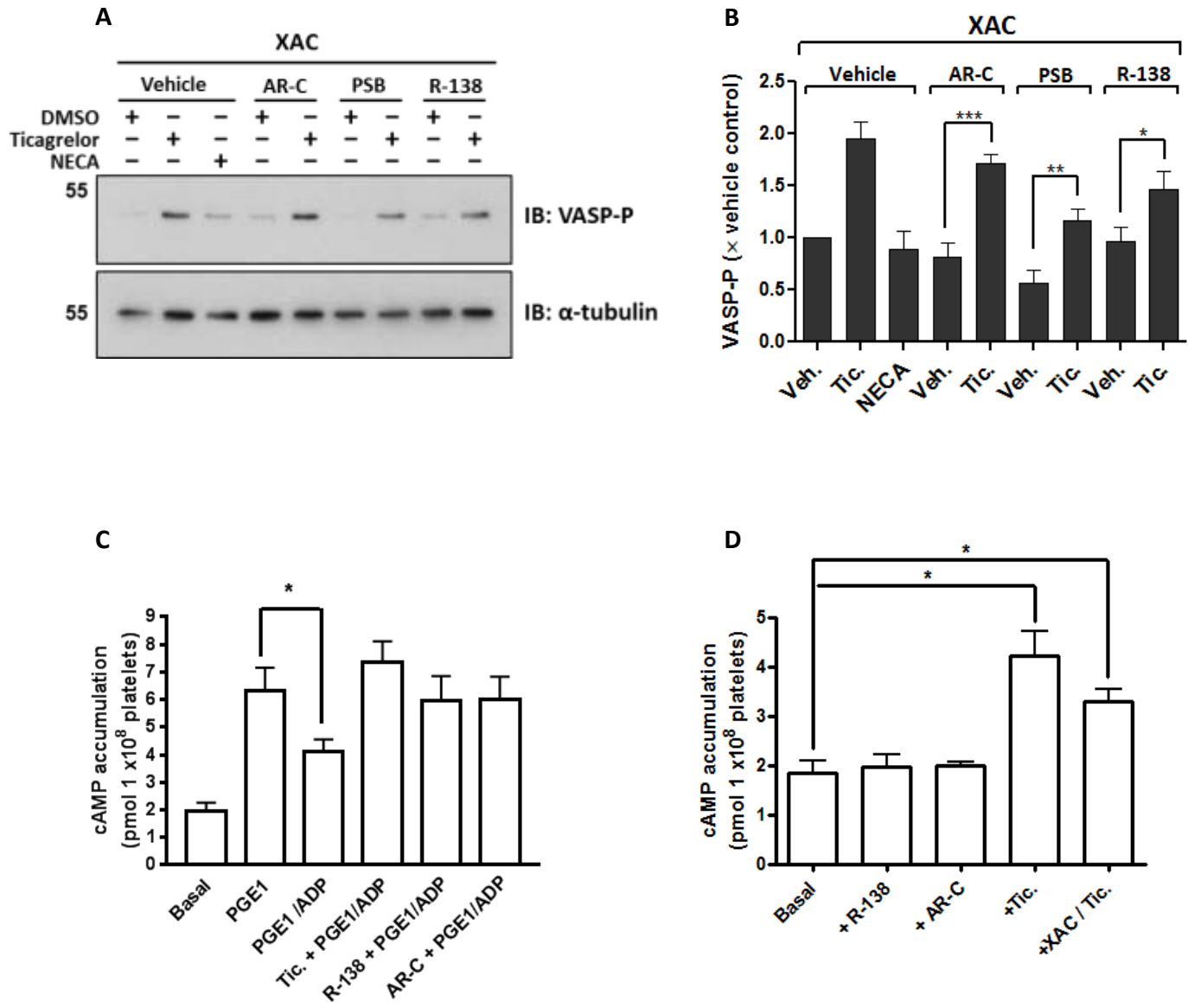


Figure 6

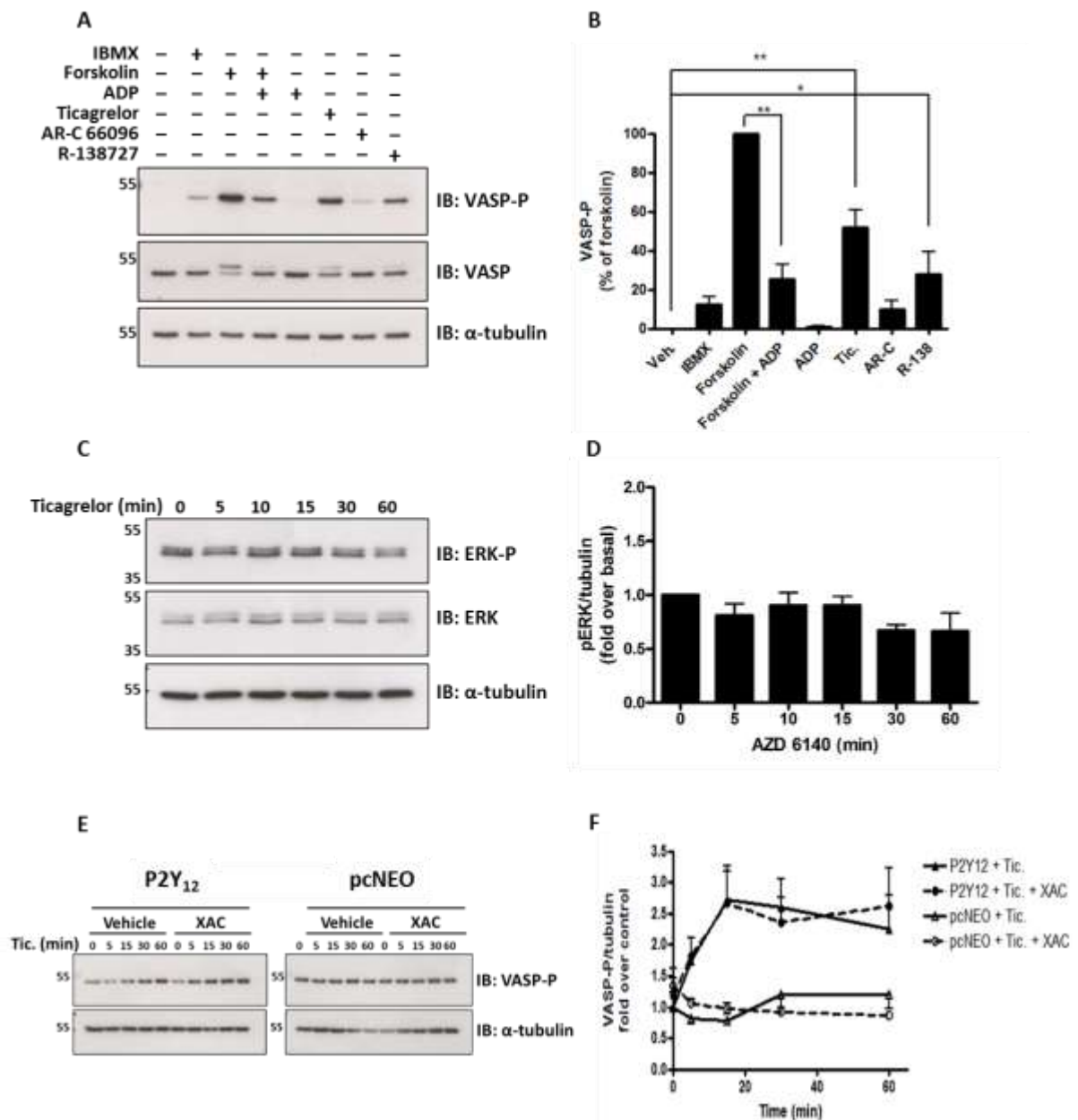


Figure 7

